AD_____

GRANT NUMBER DAMD17-94-J-4346

TITLE: Suppression of Vascular Growth in Breast Cancer

PRINCIPAL INVESTIGATOR: Luisa Iruela-Arispe, Ph.D.

CONTRACTING ORGANIZATION: Beth Israel Deaconess Medical Center
Boston, Massachusetts 02215

REPORT DATE: November 1997

TYPE OF REPORT: Annual

PREPARED FOR: Commander
U.S. Army Medical Research and Materiel Command
Fort Detrick, Frederick, Maryland 21702-5012

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. AGENCY USE ONLY *(Leave blank)*	2. REPORT DATE	3. REPORT TYPE AND DATES COVERED
	November 1997	Annual (30 Sep 96 - 29 Sep 97)

4. TITLE AND SUBTITLE
Suppression of Vascular Growth in Breast Cancer

5. FUNDING NUMBERS
DAMD17-94-J-4346

6. AUTHOR(S)
Luisa Iruela-Arispe, Ph.D.

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
Beth Israel Deaconess Medical Center
Boston, Massachusetts 02215

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING/MONITORING AGENCY NAME(S) AND ADDRESS(ES)
Commander
U.S. Army Medical Research and Materiel Command
Fort Detrick, Frederick, MD 21702-5012

10. SPONSORING/MONITORING AGENCY REPORT NUMBER

11. SUPPLEMENTARY NOTES

12a. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for public release; distribution unlimited

12b. DISTRIBUTION CODE

13. ABSTRACT *(Maximum 200*

Metastatic and primary tumor growth are both dependent on neovascularization. One can then predict that restrictions in the vascular supply to tumors could impinge on the expansion and uncontrolled growth of tumor cells. Studies presented in this proposal aim to evaluate the effect of an inhibitor of angiogenesis, thrombospondin-1 (TSP-1), in the growth of mammary tumors. The advantage of TSP-1 over other vascular inhibitors is that this protein is a natural angiogenic suppressor. TSP-1 is produced by the mammary gland during post-lactation and in fact contributes to the regression of capillaries during mammary gland involution. Therefore, we hypothesized that if effective in the regression of tumors, this molecule might not induce secondary or undesirable side effects.

We have just completed two years of studies in this proposal. Our findings are encouraging; specifically, we were able to demonstrate in the lack of TSP-1 (utilizing TSP-1 null animals), neovascularization is significantly higher than in the presence of endogenous TSP-1. Furthermore, increase in the endogenous pool of TSP-1 induces apoptosis in capillary endothelial cells. The overall effect is a reduction in the vascular supply to the mammary gland and to the highly vascularized tumors. Experiments within the next two years of this proposal will aim to understand the molecular mechanism by which TSP-1 mediates endothelial cell apoptosis.

14. SUBJECT TERMS	15. NUMBER OF PAGES
Angiogenesis, Angiogenic Inhibitors, Endothelial Cell, Thrombospondin, Vascular Biology, Capillaries, Cancer, Tumor, Extracellular Matrix, Vessels, Breast Cancer	31
	16. PRICE CODE

17. SECURITY CLASSIFICATION OF REPORT	18. SECURITY CLASSIFICATION OF THIS PAGE	19. SECURITY CLASSIFICATION OF ABSTRACT	20. LIMITATION OF ABSTRACT
Unclassified	Unclassified	Unclassified	Unlimited

FOREWORD

Opinions, interpretations, conclusions and recommendations are those of the author and are not necessarily endorsed by the U.S. Army.

N/A Where copyrighted material is quoted, permission has been obtained to use such material.

N/A Where material from documents designated for limited distribution is quoted, permission has been obtained to use the material.

N/A Citations of commercial organizations and trade names in this report do not constitute an official Department of Army endorsement or approval of the products or services of these organizations.

✓ In conducting research using animals, the investigator(s) adhered to the "Guide for the Care and Use of Laboratory Animals," prepared by the Committee on Care and Use of Laboratory Animals of the Institute of Laboratory Resources, National Research Council (NIH Publication No. 86-23, Revised 1985).

N/A For the protection of human subjects, the investigator(s) adhered to policies of applicable Federal Law 45 CFR 46.

✓ In conducting research utilizing recombinant DNA technology, the investigator(s) adhered to current guidelines promulgated by the National Institutes of Health.

✓ In the conduct of research utilizing recombinant DNA, the investigator(s) adhered to the NIH Guidelines for Research Involving Recombinant DNA Molecules.

N/A In the conduct of research involving hazardous organisms, the investigator(s) adhered to the CDC-NIH Guide for Biosafety in Microbiological and Biomedical Laboratories.

PI ~~- Signature~~ 11/7/94 Date

TABLE OF CONTENTS

INTRODUCTION

1. Original Abstract (from grant proposal)

Growth and metastasis of breast cancer is directly dependent on neovascularization. By understanding the mechanisms that control the neovascular response, it may be possible to design therapeutic strategies to selectively prevent or halt pathological growth of vessels and consequently restrain the progression of cancer cells. Despite its general biological significance and pathological relevance, relatively little is known about inhibitors of blood vessel formation. Thrombospondin-1 (TSP1), a glycoprotein originally described as a major component of platelet α-granules, has recently been identified as a negative regulator of angiogenesis. Its relevance for the suppression of vascular growth in tumors has yet to be investigated.

The present proposal was designed to address the role of TSP1 in the neovascularization of mammary tumors. Initially, kinetics of vascular development will be examined in mammary tumors of TSP1-deficient mice and in tumors of control animals. A second set of experiments will focus on the effect of TSP1 in normal and tumor-derived endothelial cells at the cellular and at the molecular level. Specifically, we will investigate the proliferation, invasion, and chemotaxis of normal and tumor-derived endothelial cells in a model of angiogenesis *in vitro*. A final facet of this proposal is directed to investigate the modulation of TSP1 receptors as both populations of cells organize into cords and tubes *in vitro* and to identify the receptor(s) responsible for the generation of signals ultimately responsible for the regulation of endothelial cell behavior in breast cancer.

2. Relevance of the present work to Breast Cancer

Partial reduction or suppression of tumorigenicity can be accomplished at multiple levels: by direct cell cycle regulation, targeted cellular ablation, control of signal transduction, and/or inhibition of angiogenesis (reviewed in refs. 1-6). Several investigators have implicated tumor suppressor genes in cell cycle regulation or signal transduction pathways and considerable effort is being made to identify the critical points in cell transformation that might be sensitive to pharmacological control. A parallel line of investigation has focused on understanding the **regulation of vascular growth in cancer.** It is recognized that an increase in the vascular supply plays a central role in tumor progression and metastasis (5-9). In fact in breast cancer, angiogenesis has been acknowledged as a significant indicator of tumor progression that is independent of axillary lymph node status (9-11). Although of recognized relevance, therapeutic approaches have generally excluded treatment of breast cancer by target ablation of neovascular growth; mostly because to date, angiogenic inhibitors have proven either too generally toxic or not selective to particular vascular beds. This proposal offers a new perspective into the concept of vascular inhibitors by focusing our attention on a natural angiogenic inhibitor present in normal mammary glands: the glycoprotein thrombospondin-1 (TSP1). According to our preliminary data, TSP1 seems to be suppressed during pathological neovascularization of breast tumors, therefore it is our premise that an exogenous supply of TSP1 should be effective and non-toxic. In addition, TSP1 seems to be specific to steroid-dependent tissues, which could potentially offer selectivity in the inhibition of mammary vessels.

3. Background and previous work done by the applicant

TSP1 has been identified as an inhibitor of angiogenesis both *in vivo* and *in vitro* (12-14). Interestingly, TSP1 has also been acknowledged as a tumor-suppressor gene in human-hamster cell hybrids and has been implicated in the inhibition of the tumorigenic ability of MCF-7 breast cancer cells (15). In addition, we recently found that TSP1 mRNA is regulated by steroids at the transcriptional level, an interesting finding considering the implication of steroids in the development of certain mammary tumors (16) Examination of the vasculature of human mammary

glands reveals consistent expression of TSP in the capillaries of normal tissue; its expression in tumors, however is not as steady. TSP1 was indeed rarely present in capillaries of the human tumors examined. Although the casual correlation between lack of TSP1 and rapid growth of tumors is attractive, whether its absence is associated with the rampant growth and capillary progression of those tumors and whether administration of TSP1 could reverse the abnormal growth of capillaries in mammary tumors remains to be tested.

Thrombospondin in the mammary gland and other steroid-dependent tissues.

High levels of TSP1 have been reported in milk, other breast secretions, and in some types of mammary cystic fluids (17). With the exception of bone and circulating platelets, the mammary gland represents one of the few adult tissues with high concentrations of TSP. Interestingly, a study on the kinetics of TSP in human milk has shown temporal variations which could be due to hormonal regulation. TSP was detected in the initial "aqueous phase" of milk secretion, its levels subsequently fell during the transition to mature milk (18). We have studied the expression of TSP1 in 32 hysterectomy cases. Our results showed stage-dependent regulation of this gene in the human endometrium (Table 1 and appendix - manuscript). Specifically we identified TSP1 protein in capillaries of the functional endometrium and this expression correlated with the end of the endometrial cycle (secretory phase). Tissues from the early proliferative phase showed no immunoreactivity. Identification of vessels with an anti-CD 34 antibody in serial sections demonstrated that only a subset of capillaries was reactive with anti-TSP-1 antibodies. Moreover, immunostaining with PCNA (proliferating cell nuclear antigen) IgG indicated that the presence of TSP-1 protein did not, in all instances, correlate with proliferating endothelial cells. Since neovascularization is also regulated by a series of inhibitory signals, we propose that TSP1 is required at later stages of the endometrial cycle to inhibit vessel formation or to stabilize newly-formed capillaries. We have also performed *in situ* hybridization on similar sections to localize TSP1 transcripts. Abundant expression of TSP1 mRNA was identified in the secretory phase, in contrast to the low levels detected in the proliferative phase. TSP1 mRNA was observed not only in endothelial cells, but also in stromal cells of the human endometrium. It was interesting that no protein was detected in stromal cells by immunocytochemistry. These observations suggest that, although stromal cells might secrete high levels of TSP1, the protein is accumulated only in the basement membranes of vessels, where it supposedly exerts its anti-angiogenic effect. Therefore, it is our presumption that TSP1 might act in a paracrine or autocrine manner to regulate vessel growth. Indeed, this hypothesis could be extended to other systems, since significant levels of TSP1 protein are also secreted by vascular smooth muscle cells (19). In the secretory phase, the distinction between the stratum functionalis and the stratum basalis with regard to TSP1 mRNA expression was striking. Whereas high levels were observed in the stratum functionalis, only background levels, equivalent to the intensity observed during the proliferative phase, were seen in the stratum basalis. These findings suggested to us that the TSP1 gene might be, at some level, regulated by steroids.

We have examined the regulation of TSP1 mRNA in cultured cells exposed to progesterone. Steady-state levels of TSP1 mRNA were elevated 4.5 fold in human stromal endometrial cells at 6h after treatment with progesterone. This effect was dose-dependent and was mediated at the transcriptional level, as shown by nuclear run-on experiments. In this context, it is interesting that analysis of the mouse and human TSP1 promoters reveal the presence of a consensus sequence (AGTCCT) (20) that has been reported to interact with the glucocorticoid receptor (21).

In the mammary gland, we have detected TSP1 in the subendothelium of blood vessels (these results were submitted as preliminary data in the grant proposal). When sections of breast cancer were examined for the presence of TSP1, we verified that capillaries were positive in some but not in all types of breast tumors.

The possibility that the TSP1 gene might be regulated by steroids has not been carefully explored, although a number of reports suggest, in an indirect manner, that this type of regulation might occur (22-24). As part of another project we have proposed to investigate this issue further to locate steroid response elements in the TSP1 gene and identify possible trans-acting factors

involved in this regulation (NIH - R29 CA65624-01). Within the context of angiogenesis, it is curious that a number of laboratories have reported inhibition of angiogenesis by steroids (25,26). It would be pertinent to evaluate whether the inhibition of blood vessel formation mediated by steroids has any correlation with the secretion of TSP1.

4. Purpose of the present work

This proposal offers a logical progression to the knowledge previously gained on the role of TSP1 in vascular biology and offers a potentially exciting avenue for the identification of a natural inhibitor of capillary growth for the treatment of human breast cancer. The successful completion of this research project will: **1.** determine whether the lack of TSP1 facilitates tumor progression and enhancement of vascular growth; **2.** identify cellular mechanism(s) by which TSP1 inhibits angiogenesis in endothelial cells; **3.** identify the receptor(s) involved in the modulation of endothelial cell behavior and examine the intracellular signaling mechanisms; **4.** determine whether TSP1 could be a selective marker for tumor-associated angiogenesis, and more importantly, **5.** determine whether the regulation of TSP1 gene can provide a natural pathway for the clinical treatment of breast cancer.

BODY

EXPERIMENTAL METHODS / AIMS

A. Is the lack of TSP1 associated with growth and metastasis of malignant tumors?

Examine the progression of the vascular bed in mammary tumors of TSP1-deficient mice.

Experimental Design/Methodology:

1. Generate mammary tumors in TSP1-deficient (tsp/tsp$^-$) mice by mating of TSP1 knock-out homozygotes with mice carrying the MMTV c-*neu* transgene

2. Analysis of the vascular bed, as well as rate of capillary extension/mm^2 of neoplastic tissue will be obtained by: a) confocal laser analysis coupled with three-dimensional reconstruction, b) determination of hemoglobin and c) endothelial cell markers. Values obtained from TSP1-deficient and from control *neu* animals will be compared.

Overall growth of the tumors and rate of metastasis will also be directly assessed and correlated with control values. Data from these experiments will concurrently provide important information on the relationship between capillary density and tumor expansion.

Determine whether exogenous TSP1 can revert/rescue the vascular phenotype of induced tumors in TSP1-deficient mice.

Experimental Design/Methodology:

1. Slow-release pellets of TSP1 protein will be implanted in the mammary fat pads of TSP1-deficient mice carrying the MMTVc-*neu* transgene.

2. Vascular progression in tumors will be determined and compared to control-*neu* mice.

3. In addition, the localization of exogenous TSP1 protein and its half life in tumors will be assessed to gain information on the fate of exogenous TSP1 in mammary tumors.

B. What are the specific effects of TSP1 on endothelial cells engaged in angiogenesis?

Investigate the specific effect(s) of TSP1 on endothelial cells engaged in the angiogenic response.

1. Endothelial cells (EC) from normal mouse mammary gland and from mammary tumors will be isolated and characterized for their proliferation rate, secretory profile, and angiogenic potential.

2. Exogenous TSP1 will be added to EC at confluence or to cells undergoing angiogenesis *in vitro*. These experiments will be performed in both tumor-derived as well as control cells. We will determine the effect of this addition on: a) proliferation; b) migration; c) chemotaxis; and d) expression of extracellular matrix-associated molecules.

Identify the cell surface receptor(s) involved in mediating cellular responses to TSP1.

1. The presence of TSP receptors will be assessed in cultures of confluent EC, as well as in angiogenic cultures, by direct binding assays.

2. Modulation of receptor number will be analyzed after addition of TSP1. 3. Neutralizing experiments with specific anti-TSP receptor antibodies will be performed to determine which of the five recognized receptors mediates an anti-angiogenic response.

RESULTS

STATEMENT OF WORK

Task 1, Examine the progression of the vascular bed in mammary tumors of TSP1-deficient mice, Months 1-24

a. Generate TSP1-deficient mice containing mammary tumors
b. Tumors from experimental and from control animals will be harvested and measured
c. Analyze the frame-work of capillaries in both experimental settings by morphometry
d. Analyze the frame-work of capillaries in both experimental settings using a biochemical strategy.

Task 2, Determine whether exogenous TSP1 can revert/rescue the vascular phenotype of induced tumors in TSP1-deficient mice, Months 12-30

a. Preliminary experiments:
Determine the rate of release of [125I]-TSP1 and determine its half life in mammary tumors. Adjust experimental conditions to accommodate these results.
b. Implant capsules containing TSP1 into the mammary tumors of TSP1-deficient mice
c. Determine the effect of TSP1 on the vascular density of mammary tumors as performed in Task #1 (c and d).

Task 3, Investigate the specific effect(s) of TSP1 on endothelial cells engaged in the angiogenic response, Months 23-40.

a. Isolate and characterize endothelial cells from normal and from tumor-containing mammary glands for their proliferation rate, secretory profile, and angiogenic potential
b. Add TSP1 to endothelial cells from normal and tumor of the mammary gland and assess a) proliferation; b) migration; c) chemotaxis; and d) expression of extracellular matrix-associated molecules.

Task 4, Identify the cell surface receptor(s) involved in mediating cellular responses to TSP1 Months 30-48.

a. Characterize and compare the spectrum of TSP1 receptors expressed by normal and tumor-derived endothelial cells at confluence and after they organize into cords and tubes.
b. Perform Western blots to determine whether one or more of the previously characterized receptors is modulated

The presence of TSP receptors will be assessed in cultures of confluent EC, as well as in angiogenic cultures, by direct binding assays. Modulation of receptor number will be analyzed after addition of TSP1. Neutralizing experiments with specific antibodies will be performed to determine which of the five receptors identified for TSP1 mediates an anti-angiogenic response.

SUMMARY OF ACCOMPLISHMENTS -

Nov. 1994 - Nov. 1996

As mentioned in the previous reports, we encountered high level of difficulty in obtaining sufficient number of animals (TSP-1 -/- */c-neu* and controls) to achieve statistical significance in task 1 and to complete task 2. Although by the end of year 2 the trend of our results was promising, we still lagged in animal number and on our ability to perform sufficient tumor studies. We are very pleased to report that this obstacle has been overpassed in year 3 (as will become apparent) .

In year 2 we also decided to use an alternative approach for task 2 (which, as originally planned, proved problematic). The alternative entailed use of stable cell lines containing TSP1 cDNA fragments driven by the CMV promoter. At the time of completion, we had concluded the selection and had tested the cell lines for expression of the truncated TSP-1 fragments, but had no results on the injection of these cell lines into nude mice. We have now (in year 3) completed this task.

In addition, in our latest report, we had described the isolation and identification of novel genes containing the anti-angiogenic domain of TSP-1. Although we have made progress in these project, related to the present proposal, we are not presenting the data here since in the past evaluation it became clear that the reviewer wanted to have major focus in the original aims of this proposal.

Also in the past critique, it became obvious that the reviewers wanted more raw data. We have therefore included twelve summary figures.

Nov. 1996 - Nov. 1997

1) We have obtained more TSP-/-/c-neu animals (total of 75 mice analyzed). This number added new data to our previous task 1. The results are presented in Figures 1 - 4

2) Task 2 was completed during this third year by using two parallel approaches:

Generation of small TSP-1 fragments that were delivered to the tumors

Generation of transgenic animals that overexpress TSP-1 to the mammary gland

Results are presented in Figures 5 - 9.

3) Task 3 has been partially concluded - We have successfully isolated endothelial cells from tumors and normal mammary glands. We have also investigated the effects of TSP-1 on endothelial cell function. Results are presented in Figures 10-11.

4) Several proposed experiments for Task 4 have also been concluded. Results are presented in Figure 12.

POINT-TO POINT DISCUSSION OF THE PROPOSED TASKS

Task 1. Examine the progression of the vascular bed in mammary tumors of TSP1-deficient mice. Months 1-24

a. Generate TSP1-deficient mice containing mammary tumors

Matings proved to be more difficult than expected for two reasons:

(1) TSP -/- animals had lower liters, therefore our starting population of animals was limited. In addition, the null mice had higher mortality and were less fertile than wild type animals;

(2) We encountered some unexpected problems in the genotype of animals, and had to verify genetic status by both Southern blot and PCR analysis, since migration of the *c-neu* band was very similar to that of the Tsp-1 band.

At this time last year we reported the following status:

A total of 69 heterozygous matings (tsp+/- / c-neu +) were set up. To improve the chances of matings, it was decided to induce ovulation in females. From all the matings we were able to obtain 14 c/neu + and tsp-/- females, as identified by Southern blot /PCR analysis. Eight of those animals developed mammary tumors by the second month.

A total of 18 animals with the genotype *c/neu* + and tsp+/+ were obtained from which 12 developed mammary tumors. In this study, we also utilized *c/neu* + and tsp-/- animals: 15 females were used at the same age as the other categories and all but one female developed mammary tumors.

Follow up matings this year greatly improved the number of animals that were analyzed for incidence and analysis of tumors. We were able to obtain 75 additional females with the phenotype tsp-/-/*c-neu* and an equivalent number of littermates with the phenotype tsp+/+ / *c-neu* . The improvement in yield related to the high number of heterozygous generated during year 2. This allowed us to successfully complete tasks 1 and 2.

b. Tumors from experimental and from control animals will be harvested and measured

Figure 1 presents data from the evaluation of 60 animals (30 each category) over the period of 10 months. We found that incidence of tumors in genetically predisposed animals occurred 2-4 weeks early in the tsp-/- background. Furthermore we observed that by 6 months of age, all *c/neu* mice in the tsp-/- background showed incidence of tumors; while this was not the case in the presence of tsp-1. In fact, 25-20% of animals in the second group remained tumor-free at the end of 10 months. The data indicates that TSP-1 is protective to the onset and incidence of genetically predisposed tumors.

In Figure 2, we show data on the evaluation of the size of tumors from *c/neu* animals on both wild-type and tsp-/- genetic background. Our results indicate that indeed, TSP-1 is important in suppressing tumor growth since its absence is permissive to an increase in tumor size of 62 to 80% over the size of tumors in the presence of this angiogenic inhibitor.

c. Analyze the frame-work of capillaries in both experimental settings by morphometry

Histological observations of the tumors revealed significant differences in the structure of the blood vessels in tumors from TSP-null animals. We have illustrated these differences in Figure 3. Macroscopically, tumors from null animals were larger, and showed bigger vascular channels than tumors from wild-type animals. Upon histological examination it became clear that the blood vessels in tsp-null tumors were larger and irregular. This was an interesting and unexpected finding that revealed the participation of TSP-1 in the morphogenesis/architecture of blood vessels.

To date, morphometrical analysis of blood vessels in tumors have been performed in six animals per experimental group at two time points: 6 and 8 months of age (were would like to have ten animals per experimental group). Evaluation included both vascular density and vascular volume. Experiments were done using thick paraffin sections (20μm). Vessels were identified by immunocytochemistry using two antibodies: anti-vWF and PECAM-1. Sections were observed and five random areas of 100μm2 were digitized using a CCD camera connected to a Nikon epifluorescent microscope. Images were quantified using NIH 1.59Image program. Three values were obtained: pixels (intensity) of nuclei staining (as revealed by DAPI staining of nuclei); pixels from vessel staining; and estimated volume of vessel staining (this last measurement was performed using a Biorad MRC-1024 confocal microscope). Total vessel density was assessed per area and normalized to total cell number. Figure 4 shows a histogram with our results to date on vascular volume. Clearly the absence of TSP-1 leads to marked increased in overall vascular volume.

d. Analyze the frame-work of capillaries in both experimental settings using a biochemical strategy.

As discussed in our previous report, this task did not work as well as originally anticipated. We had proposed to use levels of hemoglobin as a determinant of vascularization. Although we

were able to obtain measurements, the correlation between hemoglobin levels and capillary density did not hold true for tumors. Mostly because of hemorrhage in the interstitium and large, dilated and irregular vessels. Perhaps this technique could be effective as a measurement of blood vessel density in a non-pathological specimen.

We then decided to use injection of FITC-dextran to evaluate vessel volume and vascular architecture. This procedure worked well and with the aid of a Biorad Confocal microscope, we were able to obtain information that supported and supplemented the data obtained with cross sections. The main conclusions from these studies has been listed previously (Task 1, c).

Task 2, Determine whether exogenous TSP1 can revert/rescue the vascular phenotype of induced tumors in TSP1-deficient mice, Months 12-30

a. Determine the rate of release of [^{125}I]-TSP1 and determine its half life in mammary tumors. Adjust experimental conditions to accommodate these results.

1. Pilot studies on cleavage fragments of TSP1 protein

To estimate the concentration of TSP1 necessary for in vivo delivery of TSP1 and to have a clear idea of the functional activity of the different batches of TSP1 protein purified in our laboratory, we devised a functional assay. The assay consists on the ability of TSP1 protein to inhibit migration/invasion of endothelial cells into collagen gels containing bFGF. To suppress any endogenous protein, we used endothelial cells derived from tsp-/- mice and serum-free conditions. This assay has proven extremely useful to "normalize" our preparations of TSP1 and to guide our protein purification strategy to yield a greater percentage of active / functional TSP1.

For the execution of the assay, we have included heat-denatured TSP1, BSA alone, and protease-denatured TSP1. To our surprise, when we utilized thrombin-cleaved TSP1 the ability of the protein to inhibit migration was 10-15 times enhanced. Further, more refined experiments (thrombin-free) revealed that fragments of TSP-1, generated by thrombin proteolysis, were 35-50 times more potent than the intact protein in inhibiting migration of endothelial cells into collagen gels. These data would indicate that proteolysis is required, or at least enhances, the anti-angiogenic effect mediated by TSP1. They also provided new light into the possible physiological regulation of TSP-1 related to its vascular growth-suppression activity.

These results were communicated in our previous report and because of them we re-designed experiments proposed in Aim 2 to utilize thrombin-derived fragments of TSP1 (although intact TSP1 will also used).

Stability of TSP1 in the tumors was determined by injection of iodinated protein. Tumors were harvested at 5min, 30min, 1h, 3h, 5h, 12h, and 24h. The experiment was performed in 5 animals that had tumors of - 0.2 to 3cm in diameter. Breakdown of the protein was seen as soon as 30 min after injection. Complete degradation was observed by 12h in the large tumors. The cleavage of TSP1 after 30 generated two major fragments: a 140kD and a 40-50kD. These fragments were also generated after treatment of the iodinated protein with thrombin and with plasmin.

The results suggested that TSP-1 was largely unstable in tumors and that a continuous infusion of the protein will be required to maintain high levels within the tumor stroma. The results discussed together with further difficulties encountered in the diffusion of TSP1 from the pumps and the size of the pumps themselves made us reconsider the experimental design. An alternative approach, using stable transfected cells was used, instead of the pumps, to deliver recombinant TSP1 into the tumors. Although this approach entailed the generation of expression constructs and stable cell lines (which significantly delayed our timetable), it allowed us to make constructs that encoded for truncated versions of the protein that will provide further information on the mechanism of action of this protein.

b. Implant capsules containing TSP1 into the mammary tumors of TSP1-deficient mice.

As discussed above, this task, as originally designed was problematic and although four large consecutive experiments were performed, the delivery of TSP1 to the tumor was not sufficient. In

addition, the pumps were rather large for the animals and the amounts of TSP1 required to overcame rates of degradation would have been 100 fold higher than previously anticipated.

Our alternative approach entailed the use of stable cell lines containing TSP1 cDNA driven by the CMV promoter.

Four expression vectors were constructed:

(1) entire cDNA encoding TSP1

(2) a *Sac*I - *Eco*RI fragment of TSP1 encoding for a 140kD fragment

(3) a *Sac*I - *Cla*I fragment of TSP1 encoding for a 120kD fragment

(4) a construct containing the entire cDNA encoding TSP1 but containing 4 mutations in the antiangiogenic domain.

The constructs were transfected on mammary tumor cell lines (two cell lines were used: MC435 and MC 437). The selection, isolation of clones, and evaluation of protein expression was described in our latest report. During this last year we have tested these cell lines into nude mice. Figure 5 shows our results with the *Sac*I - *Cla*I fragment encoding a truncated carboxy-terminal TSP fragment that contains the anti-angiogenic domain. We compared the rate of tumor growth to that of the mutated version of this fragment. As can be seen in the figure, tumors grew slowly when cells transfected with the wild-type TSP-1 fragment in comparison to the rate seen when cells containing the mutated version of the protein were used. The mutations focused on the anti-angiogenic domain and included the following residues (underlined):

	Anti-angiogenic domain
Wild-type	**GGWSHWSPWSSCSVTCGDGVITRIT**
Mutated version	**GGWSHWSPWSSASVTAGDGLITRIT**

All other constructs proposed including TSP-1 full-lenght worked well at inhibiting tumor growth, nevertheless the *Sac*I - *Cla*I fragment was the most efficient and gave us the most impressive results.

These findings together with our previous results on the tsp-/- / c-neu mice strongly support the notion that this protein is relevant in the contention of tumor growth. We have performed analysis of tumor vasculature in tumors resulting from each construct and find that indeed the overall vascular volume is reduced (see section c).

Generations of transgenic animals that overexpress TSP-1 in the mammary gland:

To evaluate the effect of TSP-1 overexpression to the mammary gland, we also decided to follow a transgenic approach. The strategy was not originally planned in the proposal, but it was an important step to determine the overall effect of TSP-1 to the mammary gland. In particular this approach will contribute to our understanding of the biology of TSP-1 in a developing gland and will contribute to the general argument of whether TSP-1 might be an important candidate for gene therapy.

Figure 6 shows the construct that was used for the generation of the transgenic animals. Essentially the construct consisted of the MMTV-LTR promoter driving the entire coding region for TSP-1 (human) and an SV-40 polyA signal. Six transgenic lines were obtained, but only four were able to pass the transgene to the progeny. Southern analysis of the transgenic lines in Figure 6 showed that the number of copies of the transgene varied in the different lines, as can be assessed by examination of hybridization of the same blot with a TGF-ß2 gene (which is equivalent to a single copy gene).

Expression of the transgene mRNA was evaluated by RT-PCR, as shown in Figure 7. For this we used TSP-1-human specific primers to avoid detection of endogenous mouse TSP-1. GAPH primers were used as controls in all assays. In addition, we evaluated protein production by Western blot analysis. A human-specific antibody was used to provide distinction between endogenous and exogenous protein. We also assessed transgene protein by ELISA (data not shown), these results revealed an increase of 2 - 6 fold on the content of TSP-1 protein in the mammary gland.

Examination of the parenchyma by carmine staining revealed that glands from transgenic TSP-1 animals were hypoplastic. Significant underdevelopment of the glands, as indicated by lack of branching, thinner duct structures and diminished number of ducts was evident when the transgenic animals were compared to wild-type littermates (Figure 8). This was an interesting, but rather unexpected finding. There are not reports on the literature that address the effects of TSP-1 on epithelium. Interestingly, TSP-1 has been shown to be abundant in differentiated epithelial structures, including those of the mammary gland and to be a component of the epithelial basement membrane (as in capillaries).

Two interpretations were given to our finding: (1) the effects of TSP-1 overexpression in the epithelium are indirect and are caused by the reduced vascular supply, and/or, are indirect due to the insertion of transgene and disruption of genomic elements essential to epithelial morphogenesis; and (2) the effects are direct on the epithelial components and are, at least in part, independent of the effect of TSP-1 in the vasculature. We then decided to discriminate among these interpretations with the following experiments: (a) determine the structure of the parenchyma in the mammary gland of TSP null animals and (b) assess the effect of TSP-1 protein in the proliferation of mammary epithelial cells.

Figure 9 shows carmine staining of TSP-1 null and wild-type littermates. Interestingly, in contrast to the hypoplasia typical of TSP-1 overexpressors, the TSP-1 null mice showed hyperplasia and increase epithelial branching. This result provided support to the concept that TSP-1 might be influencing epithelial cells directly, since it discarded the possibility of insertional effect. It also discouraged the possibility of action through the vasculature, particularly since null animals do not show alterations (increase) in vascular density (data not shown, this is part of an NIHR29grant proposal).

We also evaluated the effects of TSP-1 on the proliferation of epithelial cells. TSP-1 has been shown to suppress endothelial cell proliferation, while is a mitogenic inducer in smooth muscle and stromal cells. Nevertheless the effects of TSP-1 on epithelial cell proliferation have not been tested. Figure 10 A shows a histogram in which the effect of TSP-1 on proliferation of endothelial cells was compared to epithelial, and stromal cells. These results indicate that TSP-1 is capable of suppressing both epithelial and endothelial cell proliferation, while the protein stimulates stromal proliferation. The effects are specific for TSP-1 since they can be reversed by two antibodies that recognize the type I repeats of the protein (Figure 10B).

Taken together these data suggest that TSP-1 might be a relevant inhibitor of tumor progression by acting in both epithelial and endothelial components of a tumor. We are currently testing the effects of TSP-1 in a variety of tumor cell lines, as well as cells isolated directly from human mammary tumors.

c. Determine the effect of TSP1 on the vascular density of mammary tumors as performed in Task #1 (c and d).

This task will be performed as previously described in Task #1(c and d). In brief, tumors were generated by subcutaneous injection of 10^7 cells (stable transfected with each construct) in nude mice. Tumors were allowed to developed for 6 weeks and their growth and vascular density was evaluated by confocal microscopy and 3-D reconstruction. The vascular volume (in μm3) of four tumors per construct was evaluated at week 6 and is presented in Figure 11 as a histogram.

As can be seen, all constructs with the wild-type form of TSP-1 were able to significantly diminish tumor vascular volume and reduce tumor size (Figure 5). A construct with the mutated form of TSP-1 angiogenic domain, however was not as proficient at suppressing tumor-induced angiogenesis.

We also evaluated the overall vascular volume in mammary glands from transgenic and wild-type littermates (Figure 12). For these experiments four animals per group were used. Four time-points were evaluated: 3, 7, 8, and 10 weeks of age. Although a clear trend in vascular volume was seen in the animal groups, i.e. lower vascular volume was seen in the transgenic animals, these differences did not appear to be statistically significant. We are now evaluating the

numbers by comparing each pair (of wild-type and transgenic littermates) independently, rather than as a group.

Task 3, Investigate the specific effect(s) of TSP1 on endothelial cells engaged in the angiogenic response, Months 23-40.

a. Isolate and characterize endothelial cells from normal and from tumor-containing mammary glands for their proliferation rate, secretory profile, and angiogenic potential

We have successfully derived a protocol for isolation of endothelial cells from mammary glands and from mammary tumors. The survival rate of these cells is relatively short (4 passages) when compared to human microvascular endothelial cells (8-10 passages). It is likely that we have not yet developed the appropriate culture conditions to keep these cells longer in vitro. Nevertheless, the number of passages is sufficient to enable us to performed the experiments planned. Essentially, in each isolation the cells are tested for the expression of endothelial cell markers and their secretory profile is evaluated. Thus far we found that endothelial cells isolated from tumors undergo program cell death more quickly than endothelial cells from normal mammary glands. Also there is no difference in the proliferation rate of endothelial cells from tumors or from normal glands. We will shortly test their angiogenic potential and evaluate their invasiveness on vitrogen and matrigel substrates.

b. Add TSP1 to endothelial cells from normal and tumor of the mammary gland and assess a) proliferation; b) migration; c) chemotaxis; and d) expression of extracellular matrix-associated molecules.

We have studied the effects of TSP-1 on proliferation extensively. Figure 10 shows that Tsp-1 is able to suppress endothelial cell proliferation in a dose-dependent manner. From Facs analysis experiments, we also know that this effect occurs in early G1 (data not shown). The effects in early G1 are corroborated by assays on pRb phosphorylation, p21 and cyclin A expression (Figure 13). When cells are plated on TSP-1 or when the protein is added to the media of synchronized cells, the effect is a delay on pRb phosphorylation and increase in p21. The overall result is cell cycle arrest in early G1. The effect on endothelial cell proliferation is more pronounced when TSP-1 is used as a substrate than when is in solution, nevertheless is effective in both states.

As a substrate TSP-1 suppresses spreading of endothelial cells, although enables adhesion. We believe that the effect of TSP-1 in cell cycle progression might be linked to the modulation on endothelial cell spreading, as well as through signaling from a TSP-1 independent receptor (as the addition of soluble TSP-1 might suggest). Interestingly, tumor endothelial cells are not as susceptible to the cell-cycle arrest when we add TSP-1 in solution. We hypothesize that variations in TSP-1 receptors might be responsible for these differences (see task 4).

Task 4, Identify the cell surface receptor(s) involved in mediating cellular responses to TSP1- Months 30-48.

a. Characterize and compare the spectrum of TSP1 receptors expressed by normal and tumor-derived endothelial cells at confluence and after they organize into cords and tubes.

b. Perform Western blots to determine whether one or more of the previously characterized receptors is modulated

This task is still in progress, but we would like to report very impressive results that dealt with the evaluation of CD-36 levels in endothelial cells isolated from tumors and from normal mammary glands (Figure 14). We found that both Western analysis and ELISA assays, the levels of CD-36 protein were significantly decreased in tumor-derived endothelial cells. The comparisons were done with three independent isolations and the blots were normalized for total protein. These data explains the mild effect on endothelial proliferation seen when proliferation assays are performed after addition of soluble TSP-1 on tumor endothelial cells. Nevertheless, cell cycle arrest is equivalent in normal versus tumor endothelial cells if TSP-1 is used as a substrate.

In any case, although the experiments described in task 3 and 4 might provide a mechanistic understanding of how TSP-1 suppresses vascular growth in tumors, it is clear from the results showed in the body of this report that this protein can prove to be an important tool for pharmacological intervention in tumors.

CONCLUSIONS

Reaching the end of year three in this project, these are our major conclusions:

1. In the absence of TSP1, incidence of mammary tumors increased significantly. These data indicate that endogenous TSP-1 is capable of suppressing early onset of tumors that originate from genetic predisposition, as it occurs in the *c-neu* mouse model.
2. The size of tumors in the absence of TSP-1 is also greater. These results reinforce the concept that tumor size is dependent on neovascularization. Furthermore they provide further support to the hypothesis that inhibition of angiogenesis in tumors can prove a strong therapy for treatment of breast cancer by suppressing tumor expansion and metastasis.
3. The number of blood vessels and overall vascular volume is significantly increased on the TSP-null background. These findings provided the first evidence that TSP-1 is an endogenous modulator of blood vessel growth.
4. The vessels in the tumors of TSP-1 -/- / *c-neu* mice were extremely dilated and less branch points were also observed. We therefore speculate that TSP-1 can provide important information to endothelial cells related to the morphogenesis and structure to the vascular network in addition to suppressing neovascularization.
5. The ability of TSP-1 to modulate neovascularization is confined to the type I repeats, as indicated in our nude mice tumor assays with stable TSP-1 cells.
6. Overexpression of TSP-1 to the mammary gland using a transgenic approach, shows that this protein affects the development of the mammary parenchyma in addition to reducing the vascular supply to the gland.
7. TSP-1 is capable of inducing cell-cycle arrest on both endothelial and epithelial cells. The suppressive effect of TSP-1 is not general since it has been shown by us and others to stimulate both stromal and smooth muscle cell proliferation.
8. The effect of TSP-1 on the proliferation of endothelial cells occurs in early G1. Our conclusions are based on experiments with pRb phosphorylation and evaluation of the cdk inhibitors p21 and p27.
9. Tumor-derived endothelial cells showed lower levels of CD-36, a receptor for TSP-1 that has been shown to bind to the type I repeats and be involved in the anti-angiogenic response.

PRESENTATIONS AND PUBLICATIONS OF FINDINGS RELATED TO THIS GRANT PROPOSAL (year 1997)

A. Presentations

The PI was an invited speaker and presented work related to this proposal in the following meetings:

1) International Symposium on Thrombosis and Haemostasis, Florence, Italy, June 1997.

2) Cardiovascular Injury, Repair and Adaptation, International Society for Heart Research, Vancouver, B.C., Canada, July 1997. Chair - Symposium: Angiogenesis in Health and Disease.

3) Gordon Conference on Angiogenesis and Microcirculation, Newport, Rhode Island, August 1997.

4) Era of Hope, Organized by the Department of Defense, Breast Cancer Research Program Meeting, Washington, DC, October, 1997. (Panel oral presentation and poster)

5) Angiogenesis and Cancer, AACR special conference, Organizers: J. Folkman and M. Klagsbrun, Florida, work will be presented in January, 1998.

B. Publications

1. Iruela-Arispe, M.L. and Dvorak, H. 1997. Angiogenesis: A dynamic balance of stimulator and inhibitors. Thromb. Haem. 78:672-677.

2. Iruela-Arispe, M.L., Ortega, M., Lawler, J., and Oikemus, S. 1997. Suppresion of vascular growth in breast tumors. Era of Hope Proceedings. 2: 347-348.

3. Ortega, M. and Iruela-Arispe, M.L. 1997. Targeted overexpression of TSP-1 in the mammary gland affects both epithelium and vasculature. (submitted).

4. Iruela-Arispe, M.L., Lane, T.F., Oikemus, S., Hynes R., and Lawler, J. 1997. Thrombospondin-1 regulates tumor-induced angiogenesis. (in preparation).

5. Iruela-Arispe, M.L., Oikemus, S., Hynes R., and Lawler, J. 1997. Thrombospondin null animals exhibit abnormal involution of blood vessels in the mammary gland. (in preparation).

FIGURE LEGENDS

Figure 1. Tumor incidence on tsp-/- and wild-type littermates that constitutively express *c-neu* proto-oncogene.
A total of 30 animals per genotype were evaluated weekly for tumor incidence. Examination was done externally by palpation and use of calipers. Animals with tumors bigger than 4cm in diameter were subjected to euthanasia.

Figure 2. Tumor size on tsp-/- and wild-type littermates that constitutively express *c-neu* proto-oncogene.
Four animals per experimental group (genotype and type point) were used for measurement of tumor size. Tumors were ressected and weighted. Histogram illustrate average size of tumors ±SD.

Figure 3. Macroscopic and histological analysis of tumors from tsp-/- and wild-type littermates that constitutively express *c-neu* proto-oncogene.
A, and **C,** - tumors from wild-type animals. **B, D, E** and **F** - tumors from tsp-/- animals. **A, B** - Macroscopic view of tumors. Note the small vessels in **A** (arrow) in contrast to the hemorrhagic appearance in **B. C-** Microscopic aspect of tumor vessels in wild-type animals. Immunolocalization of TSP-1 (arrows) is visualized in brown. **D, E** and **F-** Large vascular channels (arrows) can be seen in tumors from tsp -/- animals.

Figure 4. Assessment of vascular volume in tumors from tsp-/- and wild-type littermates that constitutively express *c-neu* proto-oncogene.
Vascular volume was determined by injection of FITC-dextran and confocal optical sectioning followed by 3-D reconstruction. Morphometric analysis of the images yielded the overall volume of the tumor vasculature expressed in μm3. Histogram represents the average of four measurements per experimental group.

Figure 5. Evaluation of tumor growth after injection of stable transfected cell lines in nude mice.
Stable transfected tumor lines containing a truncated wild-type fragment of TSP-1 (**A**) or a mutated version (**B**) were injected into the dorso of nude mice (10^7 cells/site). Subcutaneous tumors were allowed to grow and were ressected for measurement after 6 weeks.

Figure 6. Transgene construction and genomic analysis.
A. Structure of the MMTV-hTSP-1 construct. Human TSP-1 cDNA was cloned into a *Hind*III site flanked by the mouse mammary tumor virus LTR (MMTV) and the simian virus polyadenylation and splicing signal (SV40 polyA).
B. Southern analysis. Tail-derived genomic DNA was digested with *Bam*HI, blotted and hybridized to a specific SV40 polyA cDNA probe. The transgene was recognized as a 800 bp fragment. This genomic Southern includes all the founders (C, I, P, J, M and A) and the DNA of 2 wild-type mice (-). TGF-β2, unique copy number gene, was used to normalize loading and to determine the copy number of each founder (C = 1, I = 1, P = 2 and M> 10).
C. Genomic Southerns showing the progeny of 2 founders (P and M) from the first (M-A16, M-A17 and M-A18) and second generation (P-B13, P-B14, P-B15 and P-B16). The transgene copy number remained constant in the following generations.

Figure 7. Expression of the transgene.
A. RT-PCR analysis of transgene expression. RT-PCR was performed with specific primers to hTSP-1 using 1 μg of total RNA prepared from dexamethasone treated mammary glands of animals from 4 different lines. The negative control (WT) represents an RT-PCR reaction from a wild-type animal. As a positive control we used RNA from Human Mammary Endothelial Cells (HMEC) which express high levels of TSP-1. mGAPDH was used to evaluate

loading and efficiency of the PCR reaction. Arrows indicate the 886 bp hTSP-1 and 256 bp mGAPDH PCR products.

B. Western blot. Transgene protein levels were evaluated by Western analysis. Mammary protein suspension was purified by binding to heparin-sepharose beads. Concentrated proteins were fractionated by SDS-PAGE in 4-12% Bis-Tris gradient gels, transferred to nitrocellulose membranes and incubated with 1 μg/ml of anti-human TSP-1 mouse monoclonal antibody. Figure shows protein levels of eight mice, both transgenic (+) and wild-type (-), from the four different lines.

Figure 8. Morphological analysis of transgenic and wild-type mammary epithelium.
Whole-mount carmine staining of transgenic and wild-type mammary glands at 10 weeks. **A** and **E**, whole mounts from the fourth mammary gland of two wild-type animals from 2 different lines. **B** and **F**, whole mounts also from the inguinal mammary gland (4th pair) from transgenic animals belonging to the same litter. **C** and **G**, higher magnification of wild-type mammary gland whole mount from **A** and **E. D** and **H**, higher magnification of transgenic mammary gland whole-mount from **B** and **F.** Note the virtual absence of lateral branching in the transgenic mammary glands, (**B, D, F** and **H**), denoted by arrows, in comparison to wild-type (**A, C, E** and **G**).

Figure 9. Morphological analysis from glands of tsp-/- and wild-type animals.
Whole-mount carmine staining of knock-out and wild-type mammary glands at 10 week. Wild-type (**A**) and TSP-1 null glands (**B**) are shown at low magnification. **C** and **E**, and **D** and **F** shown higher magnifications of A and B respectively. Note fewer end buds in the gland from the wild-type in comparison with the knock-out (arrowheads).

Figure 10. Effect of TSP-1 on the proliferation of epithelial cells.
A. Mammary epithelial, stromal, and endothelial cells were seeded on 24well plates and allowed to spread for 4h. Minimal growth media (appropriate for each cell type) in the presence or absence of increasing amounts of TSP-1 was added to the plates. After 48h, 1μCi of [3H]-thymidine was added to each well. Fixation with TCA was done 8h later. Incorporation of [3H] -thymidine was evaluated by liquid scintillation. To allow direct comparison among cell types values were normalized to control. Each treatment was performed in quadruplicates.

B. TSP-1 (5μg/ml) alone or after was pre-incubation with a monoclonal antibody (M.ab) or a polyclonal antibody (P.ab) both at 30μg/ml was added to mammary epithelial cell cultures. Treatment was performed for 48h. As controls for the antibodies, experiments using similar concentrations of normal mouse and guinea pig IgG were done in parallel. Addition of 1μCi of [3H]-thymidine/well was done 8hours prior to the end of the experiment. Values represent the average of four wells ±SEM.

Figure 11. Assessment of vascular volume in tumors generated after injection of stable cell lines into nude mice
As previously described, vascular volume was determined by injection of FITC-dextran and confocal optical sectioning followed by 3-D reconstruction. Morphometric analysis of the images yielded the overall volume of the tumor vasculature expressed in μm3. Histogram represents the average of four measurements per experimental group. The groups include tumors from injection of: 1- cells transfected with vector alone; 2. stable lines containing full-length TSP-1; 3. stable line containing 150kD TSP-1; 4. stable line containing 120kD carboxy-terminal end of TSP1; 5. stable lines containing mutated versions of construct 4 (see description in the body of the report).

Figure 12. Vascular volume from wild-type and transgenic mammary glands.
Mammary glands at the following stages (3, 7, 8, and 10 weeks) were injected with FITC-dextran and confocal optical sectioning followed by 3-D reconstruction. Morphometric analysis of the

images yielded the overall volume of the vasculature as expressed in μm3. Histogram represents the average of four measurements per experimental group.

Figure 13. Effect of TSP-1 on endothelial cell cycle.
On the left, two photomicrographs illustrate the morphology of endothelial cells after plating on fibronectin (cells are spread) or on TSP-1 (cells are round). On the right, a series of Western blots are shown to evaluate the effect of TSP-1 on cell cycle progression. For these experiments, we used human endothelial microvascular cells. Comparison was done between cells plated on fibronectin or on TSP-1 at the following time points: 4, 16, and 30h. Note strong phosphorylation of pRb on fibronectin, but weak phosphorylation on cells plated on TSP-1. The same Western was probed with cyclin A. The upper band is non-specific (NS) and provides an idea of loading levels. The lower band (cyclin A) is only seen at 30h on fibronectin-plated cells. Expression of cyclin A, indicative of S phase, denotes that only cells on fibronectin are able to progress through the cycle. Two inhibitors, p21 and p27 were also evaluated on the same blot. Note that the levels of both inhibitors are high on TSP-1 treated cultures.

Figure 14. Expression of CD36 on endothelial cells from tumors and from normal mammary glands.
Endothelial cells were isolated from normal (N) or from tumor-bearing mammary glands (T). Equal number of cells were subjected to SDS-PAGE and the resulting Western blot was probed with an antibody to CD-36. The arrow indicates the migration and positive immunological reaction for this receptor.

REFERENCES

1. Sager, R. (1989). Tumor suppressor genes: the puzzle and the promise. Science 246, 1406-1412.
2. Bishop, J.M. (1987). The molecular genetics of cancer. Science 235, 305-311.
3. Harris, H. (1986). The genetic analysis of malignancy. J. Cell Sci. Suppl. 4, 431-444.
4. Weinberg, R.A. (1988). Finding the anti-oncogene. Sci. Am. 259, 34-41.
5. Folkman, J. (1990). What is the evidence that tumors are angiogenic-dependent? J. Natl. Cancer Inst. 82, 4-6.
6. Folkman, J. (1972). Anti-angiogenesis: new concept for therapy of solid tumors. Ann. Surg. 175, 409-416.
7. Blood, C.H. and Zetter, B.R. (1990). Tumor interactions with the vasculature: Angiogenesis and tumor metastasis. Biochim. Biophys. Acta 1032, 89-118.
8. Weidner, N. (1992). The relashionship of tumor angiogenesis and metastasis with emphasis on invasive breast carcinoma. *In* Advances in Pathology, Vol. 5, pp. 101-122, Chicago.
9. Weidner, N., Folkman, J., Pozza, F., Pierantonio, B., Allred, E.N., Moore, D.H., Meli, S. and Gasparini, G. (1992). Tumor angiogenesis: a new significant and independent prognostic indicator in early-stage breast carcinoma. J. Natl. Cancer Inst. 84, 1875-1887.
10. Weidner, N., Semple, J.P., Welch, W.R., Folkman, J. (1991). Tumor angiogenesis and metastasis-correlation in invasive breast carcinoma. N. Engl. J. Med. 324, 1-8.
11. Bosari, S., S., Lee, A.K.C., DeLellis, R.A., Wiley, B.D., Heatley, G.J., and Silverman, M.L. (1992). MIcrovessel quantitation aand prognosis in invasive breast carcinoma. 23, 755-761.
12. Good, D.J., Polverini, P.J., Rastinejad, F., Le Beau, M.M., Lemons, R.S., Frazier, W.A. and Bouck, N.P. (1990). A tumor suppressor-dependent inhibitor of angiogenesis is immunologically and functionally indistinguishable from a fragment of thrombospondin. Proc. Natl. Acad. Sci. USA 87, 6624-6628.
13. Rastinejad, F., Polverini, P.J. and Bouck, N.P. (1989). Regulation of the activity of a new inhibitor of angiogenesis by a cancer suppressor gene. Cell 56, 345-355.
14. Iruela-Arispe, M.L., Bornstein, P. and Sage, H. (1991). Thrombospondin exerts an antiangiogenic effect on tube formation by endothelial cells *in vitro*. Proc. Natl. Acad. Sci. USA 88, 5026-5030.
15. Zajchowski, D.A., Band, V., Trask, D.K., Kling, D., Connolly, J.L. and Sager, R. (1990). Suppression of tumor-forming ability and related traits in MCF-7 human breast cancer cells by fusion with immortal mammary epithelial cells. Proc. Natl. Acad. Sci. USA 87, 2314-2318.
16. Iruela-Arispe, M.L., Porter, P., Sage, H, and Bornstein, P. and Sage, H. (1996). Thrombospondin-1, an inhibitor of angiogenesis is regulated by progesterone in the human endometrium. J. Clin. Invest.*in press.*
17. Miller, W.R. and Dawes, J. (1985). Platelet-associated proteins in human breast cyst fluids. Clin. Chim. Acta 152, 37-42.
18. Drawes, J., Clezardin, P. and Pratt, D.A. (1987). Thrombospondin in milk, other breast secretions and breast tissue. Semin. Thromb. Haemostas. 13, 378-384.
19. Majack, R. A., Cook, S. C. and Bornstein, P. (1986). Control of smooth muscle cell growth by components of the extracellular matrix: autocrine role for thrombospondin. Proc. Natl. Acad. Sci. USA. 83, 9050-9054.
20. Lawler, J., Duquette, M., Ferro, P., Copeland, N.G., Gilbert, D.J. and Jenkins, N.A. (1991). Characterization of the murine thrombospondin gene. Genomics 11, 587-600.
21. Cato, A.C.B., Geisse, S., Wenz, M., Estphal, H.M. and Beato, M. (1984). The nucleotide sequences recognized by the glucocorticoid receptor in the rabbit uteroglobin gene region are located far upstream from the initiation of transcription. EMBO J. 3, 2771-2778.

22. Pratt, D.A., Miller, W.R. and Dawes, J. (1989). Thrombospondin in malignant and non-malignant breast tissue. Eur. J. Cancer Clin. Oncol. 25, 343-350.
23. Dawes, J., Clezardin, P. and Pratt, D.A. (1987). Thrombospondin in milk, other breast secretions, and breast tissue. Sem Thromb. Hemost. 13, 378-384.
24. Dreyfus, M., Dardik, R., Suh, B.S., Amsterdam, A. and Lahav, J. (1992). Differentiation-controlled synthesis and binding of thrombospondin to granulosa cells. Endocrinology 130, 2565-2570.
25. Crum, R., Szabo, S., and Folkman, J. (1985). A new class of steroids inhibits angiogenesis in the presence of heparin or a heparin fragment. Science 230, 1375-1378.
26. Ingber, D.E., Madri, J.A. and Folkman, J. (1986). A possible mechanism for inhibition of angiogenesis by angiostatic steroids: induction of capillary basement membrane dissolution. Endocrinology 119, 1768-1775.

List of Personnel involved in this project
For year 3 - 10/96 to 10/97

Dr. Luisa Iruela-Arispe	- Principal Investigator - 20%
Dr. Mariasun Ortega	- Post-doctoral fellow - 100%
Ms. Sarah Oikemus	- Research Assitant - 40%

Figure 1.

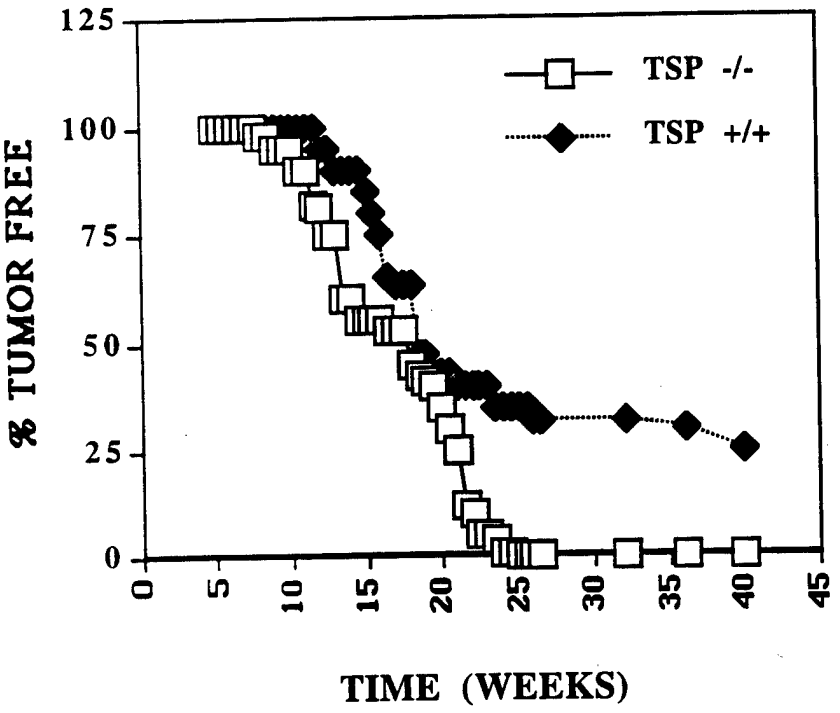


Figure 2.

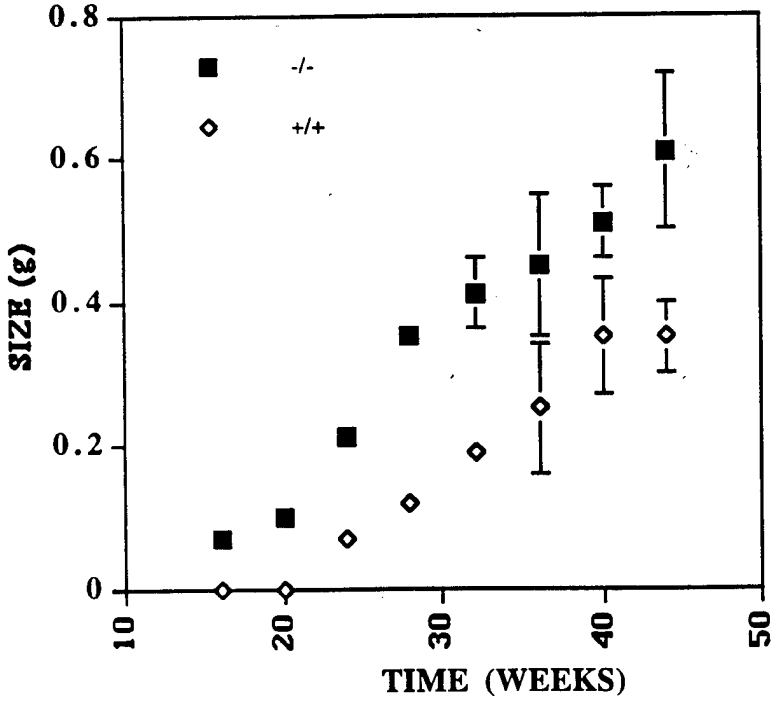


Figure 3.

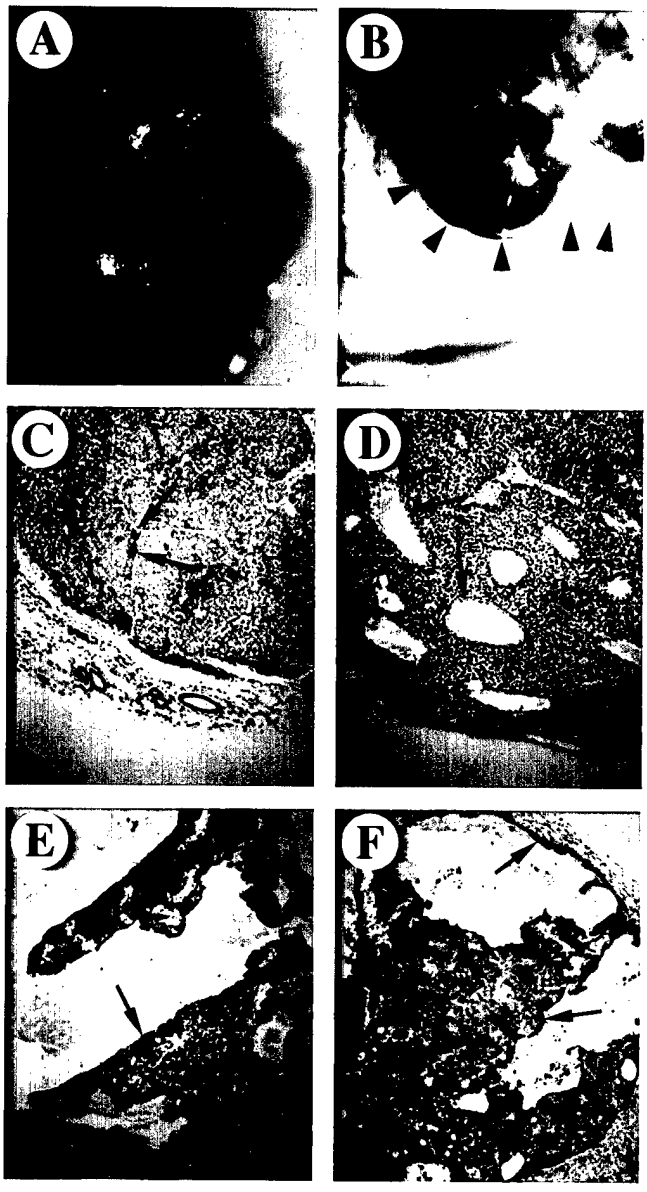


Figure 4.

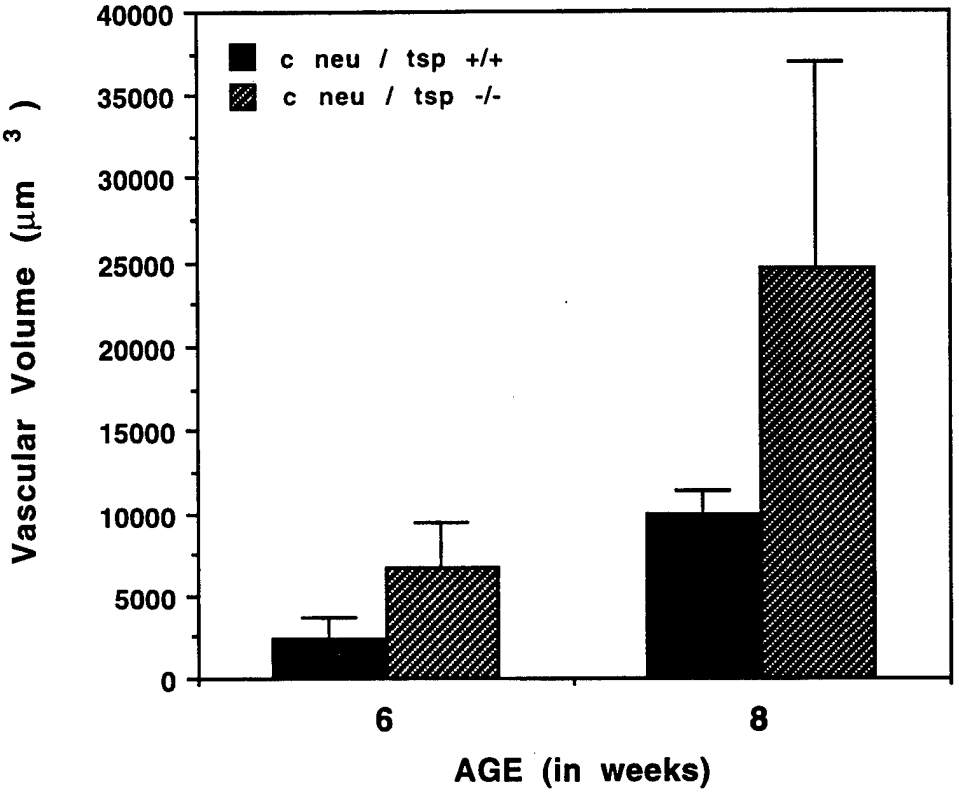


Figure 5.

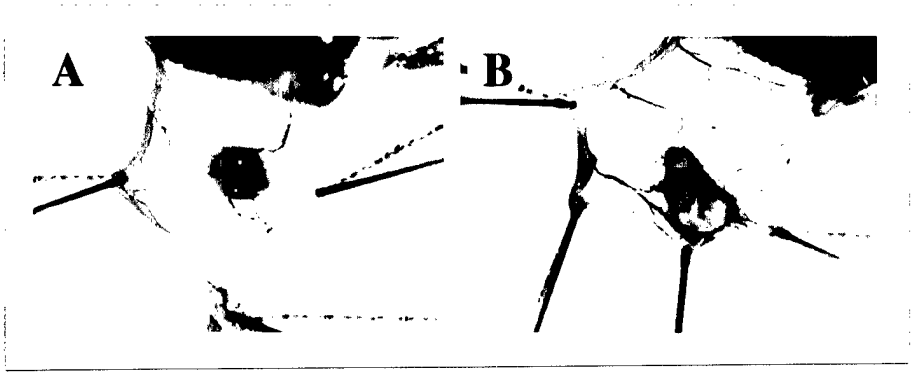


Figure 6.

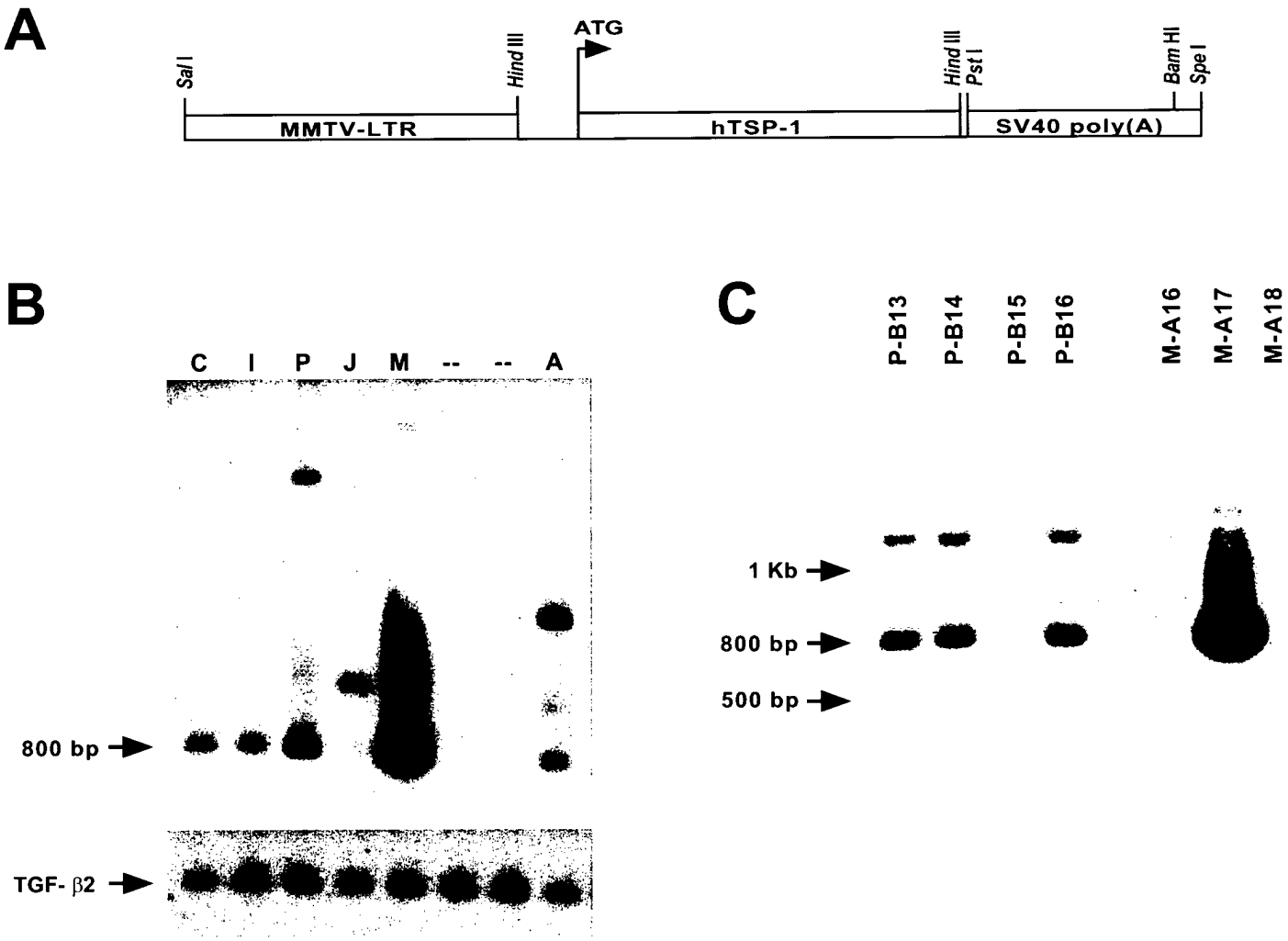

A
Sal I
Hind III
ATG
Hind III
Pst I
Bam HI
Spe I
MMTV-LTR
hTSP-1
SV40 poly(A)
B
C I P J M -- -- A
800 bp
TGF- β2
C
P-B13
P-B14
P-B15
P-B16
M-A16
M-A17
M-A18
1 Kb
800 bp
500 bp

Figure 7.

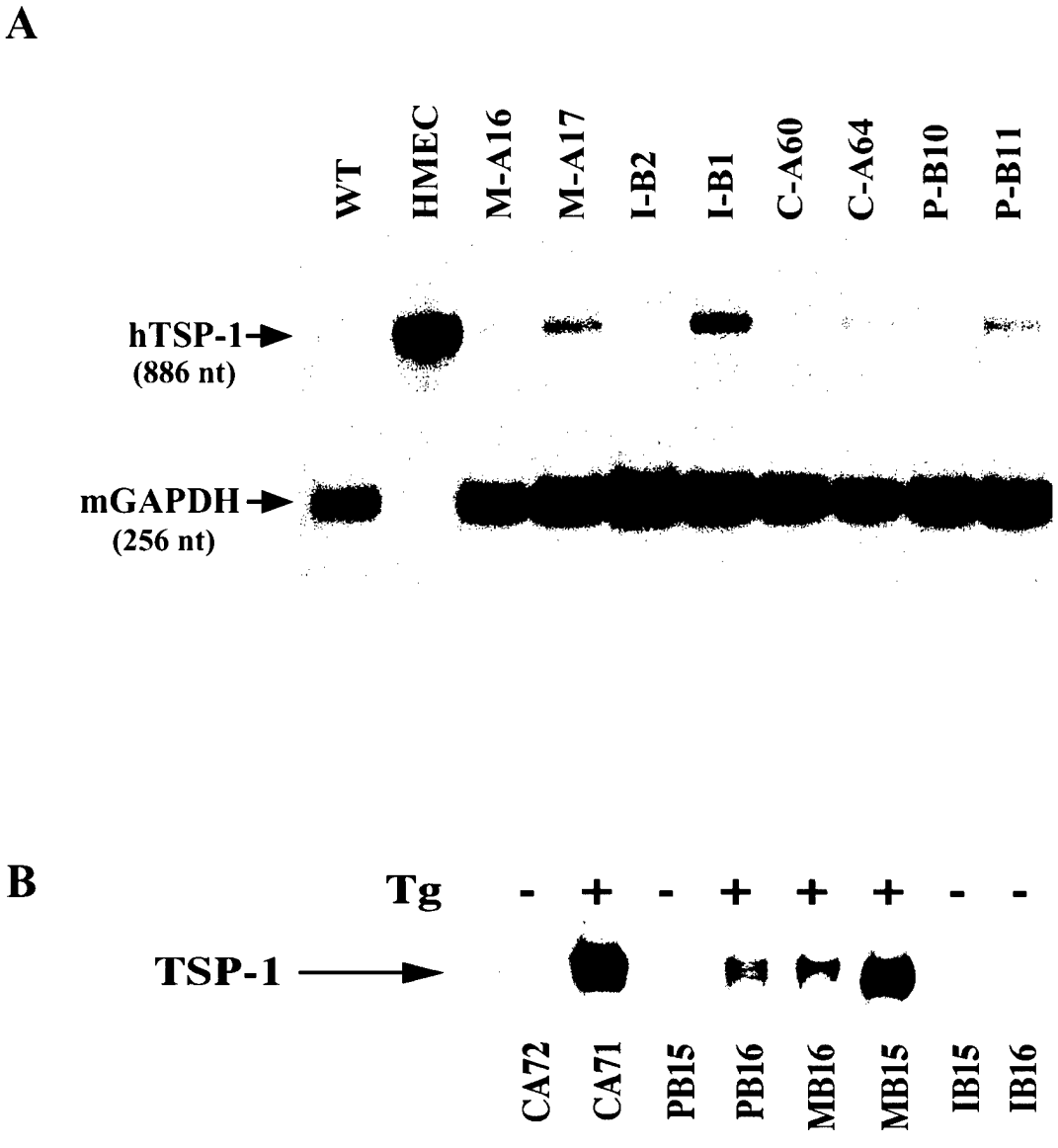

A
WT
HMEC
M-A16
M-A17
I-B2
I-B1
C-A60
C-A64
P-B10
P-B11
hTSP-1
(886 nt)
mGAPDH
(256 nt)
B
Tg
- + - + + + - -
TSP-1
CA72
CA71
PB15
PB16
MB16
MB15
IB15
IB16

Figure 8.

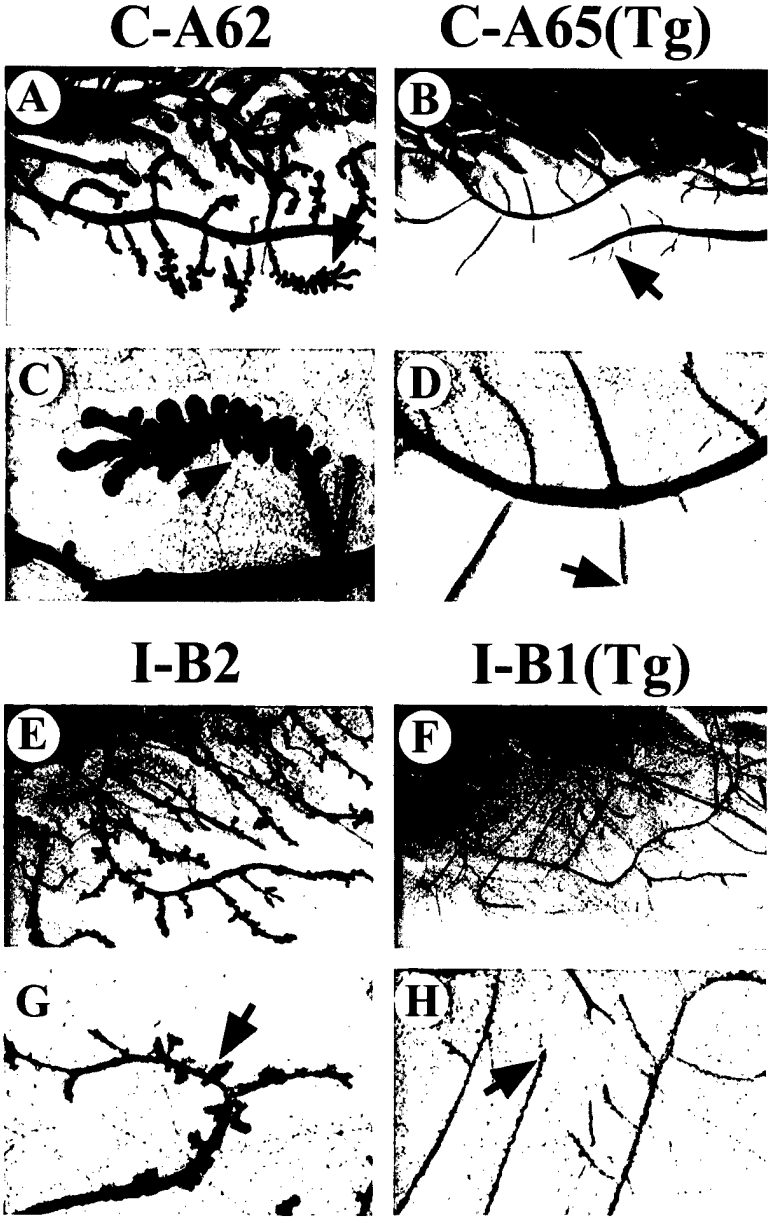


Figure 9.

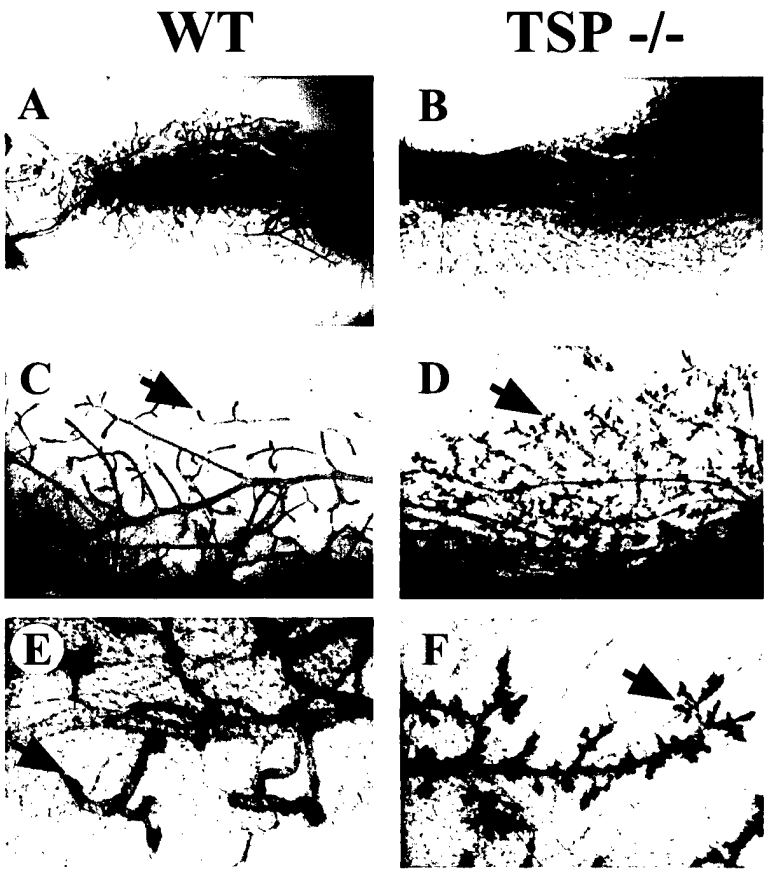
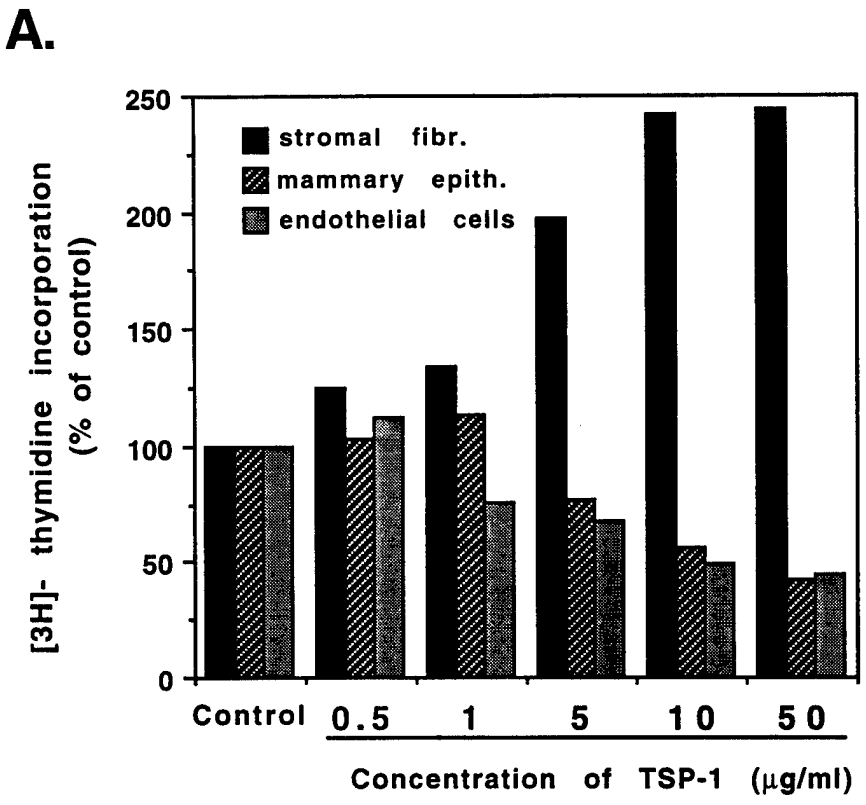


Figure 10.


A.
250
200
150
100
50
0
stromal fibr.
mammary epith.
endothelial cells
[3H]- thymidine incorporation
(% of control)
Control
0.5
1
5
10
50
Concentration of TSP-1 (µg/ml)

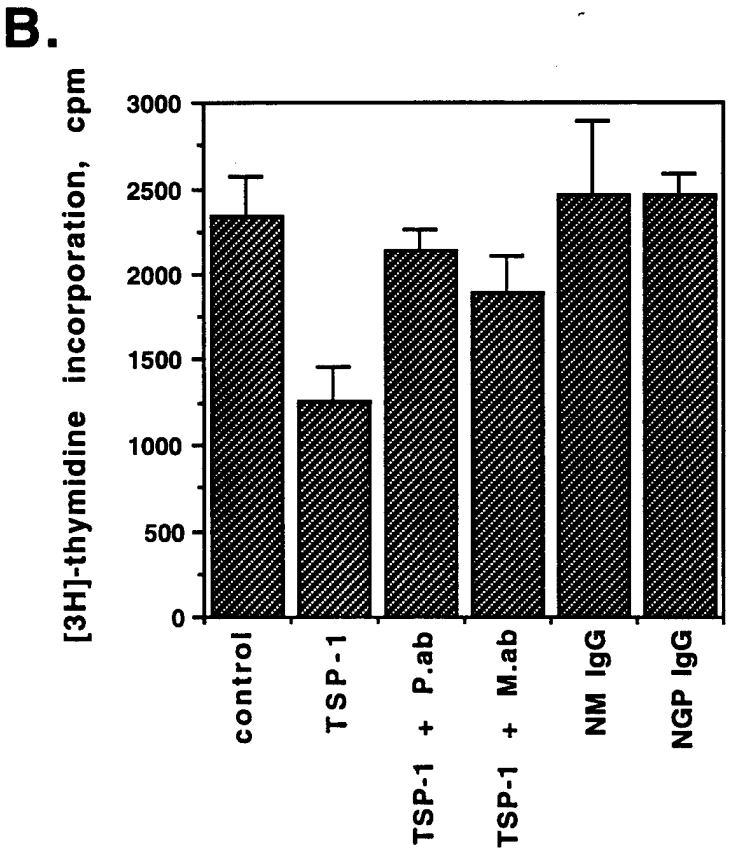

B.
3000
2500
2000
1500
1000
500
0
[3H]-thymidine incorporation, cpm
control
TSP-1
TSP-1 + P.ab
TSP-1 + M.ab
NM IgG
NGP IgG

Figure 11.

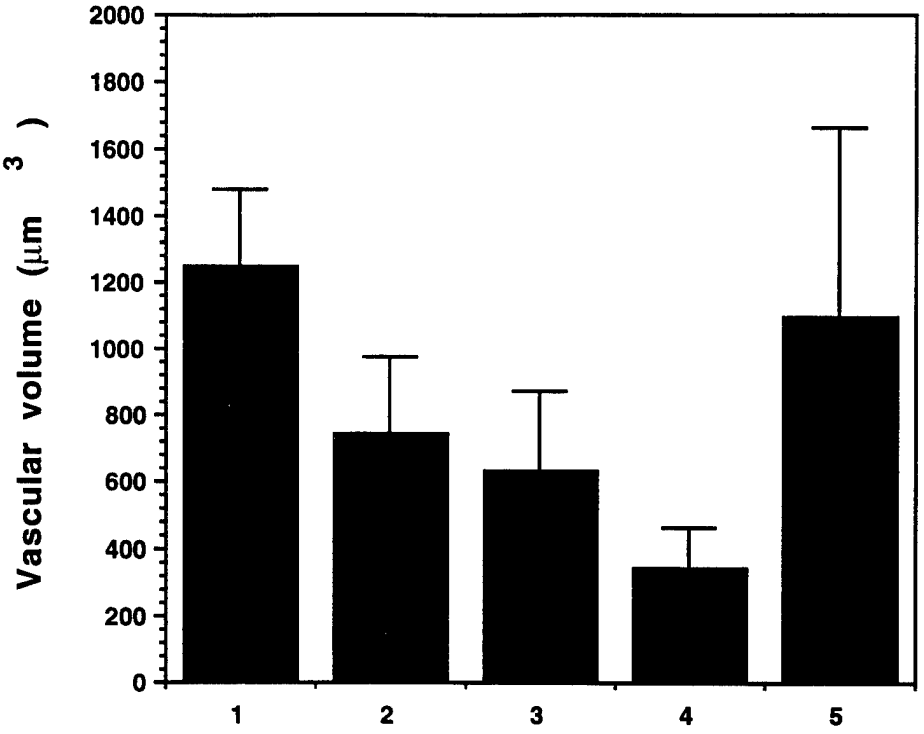


Figure 12.

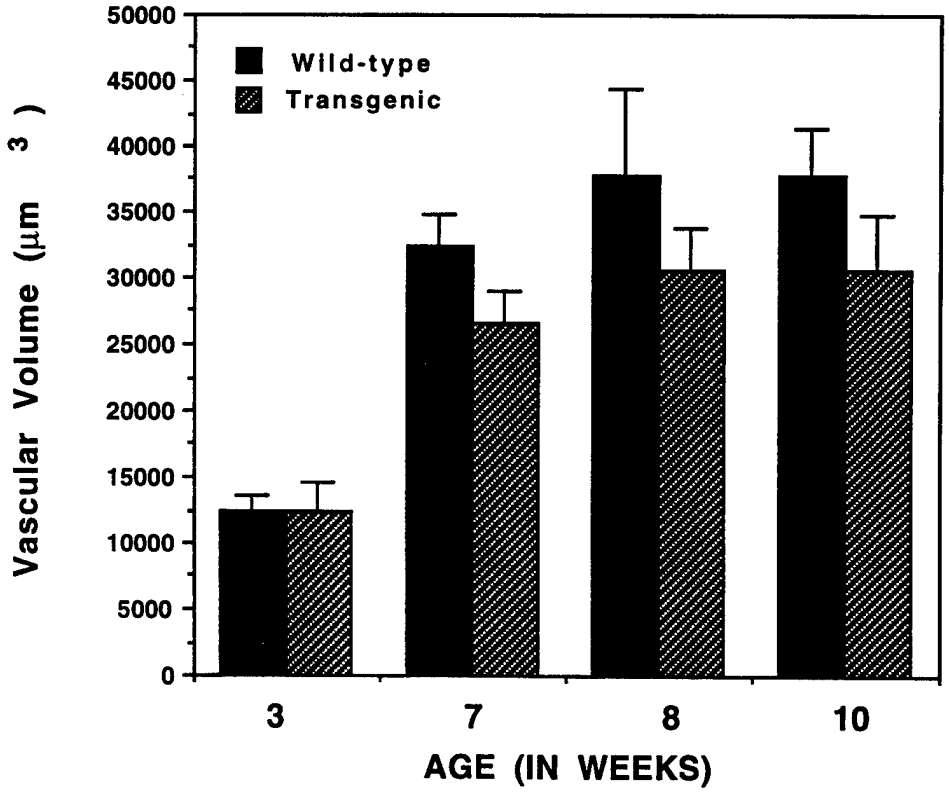


FIGURE 13

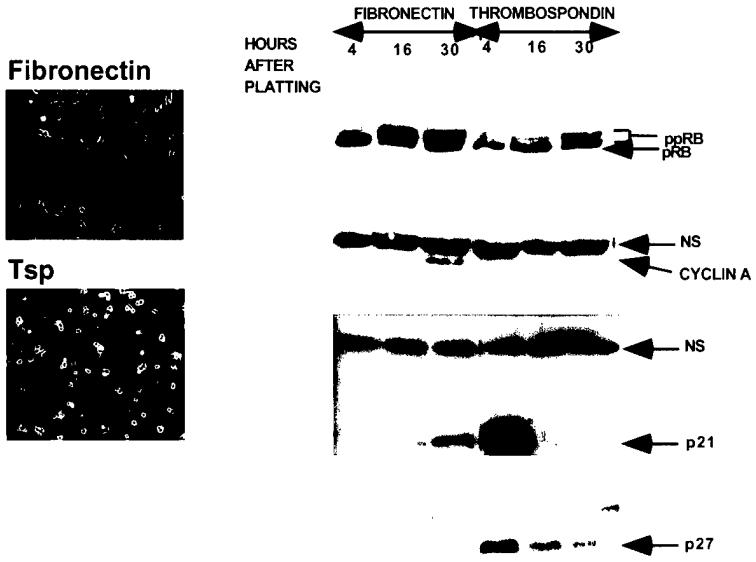


FIGURE 14